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REVIEW

# Immunophenotyping of acute leukemia and lymphoproliferative disorders: a consensus proposal of the European LeukemiaNet Work Package 10

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**The European LeukemiaNet (ELN), workpackage 10 (WP10) was designed to deal with diagnosis matters using morphology and immunophenotyping. This group aimed at establishing a consensus on the required reagents for proper immunophenotyping of acute leukemia and lymphoproliferative disorders. Animated discussions within WP10, together with the application of the Delphi method of proposals circulation, quickly led to post-consensual immunophenotyping panels for disorders on the ELN website. In this report, we established a comprehensive description of these panels, both mandatory and complementary, for both types of clinical conditions. The reason for using each marker, sustained by relevant literature information, is provided in detail. With the constant development of immunophenotyping techniques in flow cytometry and related software, this work aims at providing useful guidelines to perform the most pertinent exploration at diagnosis and for follow-up, with the best cost benefit in diseases, the treatment of which has a strong impact on health systems.**
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## Introduction

As soon as the European LeukemiaNet (ELN) project started, the 'Diagnostics' workpackage (WP), WP10, initiated a discussion aiming at obtaining a state-of-the-art view on the immunophenotyping step of leukemia diagnosis. An electronic document, mostly based on Delphi consultation of a large number of partners in the WP and on discussions during a few meetings, was posted at the ELN website by early 2005, listing the antigens considered mandatory to investigate and providing an additional list of potentially useful markers.[1] Two other documents, respectively, considering pre-analytical steps and proposing a series of panel combinations of reagents validated in individual laboratories, were posted consecutively.

After 5 years, it appeared necessary to revise the 'mandatory' panel and its appendix, and to provide a more explicit document, which would facilitate data interpretation. In accordance with WP10's philosophy, this paper was therefore submitted to and reached consensus from the WP's participants. It presents the state-of-the-art and a global guideline to immunophenotyping in acute leukemias (ALs) and lymphoproliferative disorders (LPDs), also delineating the information provided by the selected markers and their value at diagnosis. This document deliberately does not enter the fields of panel construction or minimal residual disease (MRD), discussed elsewhere by this group of laboratory experts. It aims at better assessing the value brought by individual differentiation antigen recognition in properly delineating hematological malignancies.

An important characteristic of proper immunophenotyping of hematological malignancies at diagnosis is that it should provide all relevant indications to the clinician, including classification of the disease, according to maturation arrest stages[1–5] and pertinent information for the immunophenotypic follow-up of each patient.[6] The complexity and diversity of leukemias and LPDs are indeed associated with a large variety of molecular and immunophenotypic anomalies identified within the integrated approach recommended by the World Health Organization (WHO), which was considered in detail when writing this paper.[7] Indeed, the bases of AL immunophenotyping were summarized and typified in the European Group for the Immunological Characterization of Leukemias classification,[2] from which the immunophenotyping of AL was developed by the WHO classification of 2008.[7,8] Immunophenotyping has become a major diagnostic tool in addition to morphology, owing to its accuracy and speed.[8] Cellular biomarkers readily detected through immunophenotyping allow a proper definition of hematological malignancies' lineage and differentiation, whereas cytogenetic or molecular anomalies further characterize subsets of these diseases sometimes belonging to the same immunophenotypic family.[8] The pertinent choice of a rather large panel of antibodies is therefore required at diagnosis to

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best orient further molecular studies, and, more importantly, the therapeutic options for each patient.

Both for AL and LPD, the ELN proposals consist of two parts. The first part of the panel is considered mandatory, but will not be sufficient for a final diagnosis in most cases. A selection of markers from the second panel will often be necessary to finalize the diagnosis, in the context of the integrated approach recommended by the WHO,[7,8] considering not only morphology and immunophenotyping, but also all clinical and laboratory information available. Indications as to the usefulness and relevance of all markers proposed here to identify abnormal cells are provided and referenced.

## AL immunophenotyping

In the vast majority of cases, clinical and cytological information will be available by the time a sample is forwarded for immunophenotyping. Nonetheless, the panels should be constructed to allow not only lineage assessment using just a few markers, but also comprise enough antibodies to apply existing classifications[1,7,9] and detect immunophenotypic features helpful for later investigation of MRD, such as co-expression patterns and potential aberrant expressions, although this is not the topic of this paper and has recently been reviewed elsewhere.[6] Former biphenotypic AL,[2] now renamed mixed phenotype AL,[7] although rare, should not escape such a proper investigation, nor should other rare forms of leukemias mentioned below.

It has been recognized that within the two major groups of leukemias, derived from the lymphoid or myeloid lineages, the following entities can be identified through proper immunophenotyping:[7]

### *Acute lymphoblastic leukemia (ALL)*

- B lineage: B-I, B-II, B-III, B-IV,[2,10] where, in addition to the degree of differentiation of B-lineage lymphoblastic ALs, the differentiation between B-precursor AL and Burkitt's lymphoma/leukemia is necessary for important therapeutic consequences (Supplementary Table 1).
- T lineage: T-I, T-II, T-III, T-IV.[11]

### *Acute myeloblastic leukemia (AML)[9]*

- With minimal differentiation (former M0).[12]
- AML with granulocytic or monocytic differentiation.[13]

### *Acute promyelocytic leukemia (APL)[14]*

- Erythroid.[15]
- Megakaryocytic.[16]
- Dendritic cell (DC) precursors.[17–19]
- Basophils and mast cell precursors.[20,21]

Rare acute undifferentiated leukemias express no markers considered specific for myeloid or lymphoid lineage,[7] and usually display the immature immunophenotype $CD34^{+}/CD45^{dim}$. Mixed phenotype AL show either differentiation towards both myeloid and lymphoid lineage in a single blast population or two separate, myeloid and lymphoid blast populations.[7]

The panels used should also allow for a differential diagnosis between AL, lymphoma, non-hematological neoplasms and reactive cytopenias, for example, following drug administration.

## Mandatory panel

The consensual mandatory AL diagnostic panel comprises 27 antigens as shown in Supplementary Table 2 issued from the electronic document posted on the ELN website.[1]

The first set of markers is stated to be used 'for quick orientation or paucicellular samples'. The former statement refers to samples arriving outside normal working hours when the clinicians, nonetheless, need a rapid answer to initiate therapy. This is a rare and exceptional occurrence, as in most patients treatment initiation can be delayed by a couple of days, especially if enrollment in a clinical trial is considered. Immunophenotyping therefore can be planned in advance and the sample forwarded to the laboratory with ample time to provide results by the end of the day, which by the way is one of the strong points of this technology. In some cases with major fibrosis, it will however appear difficult to obtain an adequate bone marrow specimen for a complete panel and immunophenotyping may be performed *a minima*. In such cases, a complete panel should be performed on a blood sample as leukemic cells are usually present also in the periphery, although sometimes at low frequency. Technological advances in multiparameter flow cytometry in the past few years allow for both more comprehensive immunophenotypic investigations on small samples and the characterization of small contingents of abnormal cells together with useful investigations of co-expressed markers. This is especially true and accurate through the now widely used addition of CD45 staining in all combinations.[22,23] The CD45/right angle (or side scatter, SSC), displayed in Supplementary Figure 1 (top panel), can be considered as a 'map' of the studied sample. Specific combinations positively identifying mature monocytes, granulocytes and lymphocytes as well as $CD45^{-}$ erythropoietic precursors[24,25] can be used to define the respective location of these cells on such a reference 'map'. The remaining low SSC/low-intermediate CD45 area obtained by excluding these mature cell subsets, dubbed the 'bermudes' area by the GTLLF (Groupe de Travail sur les Leucémieset Lymphomes Francophone),[25] comprises immature hematopoietic cells and the blast population can be found there in the vast majority of AL cases. This CD45/SSC scattergram can be very useful to refine the identification of the blast population by applying backgating on it, that is, superimposing on this scattergram cells expressing another marker or marker combination by using a different coloring (Supplementary Figure 1, bottom panels in normal bone marrow). Knowledge of the normal expression pattern of tested markers in remaining normal cell populations is very useful as a technical internal control of the labellings. An atlas of flow cytometry patterns found in normal bone marrow has been posted at the ELN website[26] and can be referred to. With the development of multidimensional flow cytometry and analysis methods allowing a better definition of the blast cell population, the classical thresholds of 20 or 10% of positive cells[2] have become less relevant. It is more important to assess the fluorescence shift and pattern of the whole clonal leukemic population, by comparison with fluorescence intensity of the tested marker on negative cells in the same sample.[27]

The first set of proposed markers: cytoplasmic CD3 (cCD3), myeloperoxidase (MPO), cCD79a and TdT include lineage markers with a cytoplasmic or nuclear localization. It will therefore require cell permeabilization reagents.[28] Cytoplasmic expression of CD3 or CD79a is one of the earliest events occurring upon commitment of a progenitor cell towards the T or B lymphoid lineage, respectively. In normal differentiation, these antigens will be later used to bring the antigen receptor

(T-cell receptor or B-cell receptor) on the surface of the mature lymphocyte. Therefore, these early markers characterize the T or B lineage of ALL. However, it has to be noted that some T-precursor ALLs have been shown to express cCD79a weakly.[29] The MPO enzyme, which can easily be identified with specific antibodies, is conversely the hallmark of myeloid cells, and hence is expressed in most AML, but absent in lymphoid cells. However, AML with minimal differentiation and some monocytic AMLs are $MPO^-$.

TdT, the terminal deoxyribonucleotidyl transferase, is a DNA-repairing enzyme almost always present in the nucleus of B- and T-precursor ALL blasts and in some immature AML cases, but absent from lymphoma cells (except for rare precursor B and T lymphomas), and is therefore useful for differential diagnosis between these two entities.

The second set of differentiation antigens aims at confirming the diagnostic orientation provided by intracellular staining. It has been proposed that at least two differentiation antigens from the same lineage should be present on the blast cells to confirm their commitment. CD7 and CD2, which are early differentiation molecules appearing during the bone-marrow stage of T-cell maturation that remain all through T cells' life, are therefore proposed there. However, CD7 is often absent in mature T-cell lymphomas, especially adult T-cell leukemia lymphoma and Sezary syndrome.[30] Of note, these two antigens can be expressed in AML, although seldom concomitantly.

The immunophenotyping of T-lineage blasts should also include CD5, which is another early differentiation antigen, CD1a for the identification of corticothymocyte-like T-III ALL and the MHC ligands CD4 and CD8. The latter are absent in the most immature forms of T-ALL, can be co-expressed as in the double-positive stage normally present during thymic differentiation and become mutually exclusive in more mature forms. It is also important to check for the usual absence of surface expression of CD3 in T-ALL. The presence of this molecule on the membrane characterizes the more mature $CD1a^-$ T-IV ALL.

During B-cell differentiation, cytoplasmic expression of CD22 appears early, quickly followed by translation of the molecule to the cells' surface. CD19 is one of the first differentiation antigens of the B lineage, and according to WHO 2008, CD19 expression is required for B-lineage assignment together with one (if strongly expressed) or two (in case of dim CD19) other B-lineage antigens. However, caution should be exerted when CD19 alone is observed, as this molecule displays broad lineage promiscuity and can be seen in AML.[31] CD22 alone is seen in AML cases with basophilic, mast cell or dendritic differentiation.[32] Assessment of surface immunoglobulin expression is an important feature to identify or rule out mature Burkitt's lymphoma B cells. CD10 is the hallmark of childhood most common ALL, and was indeed initially called CALLA for common ALL antigen.[33] It will therefore confirm the B lineage of most childhood and many adult ALL. Its absence, in cases expressing other B-lineage markers, may indicate the poorer prognosis B-I ALL. However, CD10 is also found in many cases of B-III ALL, mature B-cell lymphomas, including Burkitt's lymphomas and in some T-ALL. Within the B lineage, it is also important to investigate for the potential presence of cytoplasmic immunoglobulin μ-chains, signing when expressed in the absence of surface immunoglobulins the pre-B stage of B-III ALL. In normal differentiation, this stage indicates that a proper rearrangement of the variable domain of the IGH gene coding for immunoglobulin heavy chain has been achieved. The corresponding peptide (variable domain) together with the constant domains of IgM μ-chain is produced and accumulates in the cytoplasm. Minute amounts of these IgM chains also appear on the surface of the cell with surrogate light chains and constitute the pre-BCR.[34] This maturation step allows for the initiation of VJ rearrangement on one of the light-chain genes.

Finally, the two myeloid-lineage-associated markers CD13 and CD33, usually together with MPO, but in the absence of lymphoid-lineage-associated markers, would allow to confirm or identify an AML.

CD34 is an immaturity marker, present on stem cells and often still expressed by leukemic blasts. In the extremely rare cases of acute undifferentiated leukemia,[8] its presence as only differentiation antigen will, together with weak positivity for CD45, confirm the hematopoietic origin of the tumor cells.

Expression of the class II HLA antigen DR, also a hallmark of stem cells, can be helpful for the identification of acute undifferentiated leukemias, but is also almost always present on B-ALL cells and often in AML, except promyelocytic M3 AML (APL).[14] It is also expressed by normal B cells, monocytes and activated T cells.

The comprehensive mandatory immunophenotyping panel recommended here to be performed in a single step for all diagnoses comprises a further set of 13 markers. The immature myeloid marker CD117 is found in about 75% of AML blocked at an early step of differentiation.[35] CD14 is seldom observed on AML blasts, but can be present in some rare cases with myelomonocytic differentiation. However, CD14 is useful to properly separate blast cells from remaining monocytes as the latter, in addition to CD14, co-express physiologically CD33 and CD13, a feature also often seen on AML blasts. It is of note that flow cytometry is much more accurate than morphology or other techniques to dissect monocytic differentiation, by identifying through immunophenotypic pattern the consecutive stages of monocytic maturation.[1]

CD65 is more characteristic of blasts engaged in granulocytic differentiation, appears earlier and seems to be more pertinent than the other carbohydrate differentiation antigen CD15. The latter, but not CD65, displays lineage promiscuity and can often be seen in some cases of B-lineage ALL of the B-I type,[36] as well as during myelomonocytic differentiation, together with CD65.

CD56, the neural cell adhesion molecule, normally expressed on some neural cells, natural killer (NK) lymphocytes and sometimes on activated monocytes, will be found as nearly sole marker on lineage-negative precursor DC leukemias,[18] usually together with surface CD4. However, this phenotype is not systematically found among plasmacytoid DC neoplasias and other more specific plasmacytoid DC markers should be tested in such cases. CD56 is often expressed on AML blasts and in these cases does not indicate NK-cell differentiation.

The identification or exclusion of megakaryocytic leukemias can be achieved by testing the platelet integrins CD41/gpIIb or CD61/gpIIIa, either on the surface or in the cytoplasm of blasts. For the rare leukemias of erythroblastic lineage, a red blood cell marker such as CD235 (glycophorin A) or CD36 (thrombospondin receptor) can be used. The latter will also stain platelets and monocytes. However, platelets often adhere to blast cells in AML and false-positive results may also be obtained with CD41 and CD61 antibodies.[37] Backgating of the stained population on an SSC/CD45 scattergram will help in such cases. Care should also be taken in choosing the red blood cell lysis reagent, as some may alter also nucleated red cell precursors.

## Useful additional markers

In B-lineage ALL, cytoplasmic or surface immunoglobulin light-chain expression can be investigated. It will be negative except in the most mature forms of blast cells (B-IV), often related to

Burkitt's lymphoma, thereby confirming clonality. Care must be taken to work on washed samples for this assay, as light chains are present on plasma immunoglobulins that must and should be the first to be removed. Cytoplasmic expression of light chains may also be the only marker of the rare surface immunoglobulin-negative B-IV ALL.[38] The tetraspanin CD9 is also often associated to the B lineage and can be used as a confirmatory marker. It has been reported to be predictive of TEL/AML1,[39] but can also be found on variant APL/M3 cases. Another important molecule involved in B-cell differentiation is PAX5 that regulates CD19 expression. Antibodies to PAX5 have been developed that are efficient on bone marrow biopsies, but limited information is available on their use in flow cytometry. However, this protein has also been reported to be expressed in acute myeloblastic leukemias with the t(8;21), often also expressing CD19.[40]

For T-lineage ALLs, expression of the T-cell receptor can also be investigated in the cytoplasm and/or on the blasts' surface for discrimination between T-IVa and T-IVb.[41]

In AML, a more refined exploration of the enzymatic content of the cells may be useful to assess their maturation level. MPO is the first enzyme to appear during myeloid differentiation, whereas lactoferrin, hallmark of the late neutrophil granulocyte compartment,[42] is only present on the most differentiated AML.[43,44] Lysozyme will conversely identify early monocytic differentiation[45] and discriminate negative plasmacytoid DCs from positive myeloid cells.[46] CD133 is an alternative marker for progenitor cells. CD34 and CD133 have a broad overlap in the reactivity of progenitor cells. CD133 can be used for the therapeutic preparation of stem cells and it should therefore be evaluated on AL blast cells in such cases.[47]

The integrin α-chains CD11b and CD11c are frequently expressed in AML, but are absent from APL.[48] These M3 AML, which display the t(15;17) translocation fusing the *PML* and *RARA* genes, also usually lack CD34 and HLA-DR.[14] Positivity for CD34 in APL has been reported to be associated with the hypogranular variant and may confer worse prognosis.[49]

The carbohydrate antigen CD15, already mentioned above, can be a useful complement for the identification of AML with granulocytic or monocytic differentiation.[14,28] It often is expressed as a gradient even on apparently otherwise immunophenotypically uniform blasts. CD15 may also be useful to exclude remaining mature neutrophils or identify hemodilution of a bone marrow sample. As a complement of the distinction of the blast population on a CD45/SSC scattergam, as described above, the appreciation of CD16 (Fc-γ RIII) and CD11b expression qualifying mature granulocytes can be tested. CD11b and CD64 (Fc-γ RI) will, moreover, further identify the monocytic compartment.[27,50]

CD35 expression, often associated with that of CD36 on less immature AML cells, has been reported to segregate a clinically relevant subset of AML.[9]

CD71, the transferrin receptor, is an activation marker expressed in a large fraction of AL. Taken together with CD36, it is also useful for the characterization of erythroblasts, either normal and attesting of residual hematopoietic activity or consisting of the M6 blast cell population.[51,52]

Bright cytoplasmic expression of the mucosialin CD68 is characteristic of most AML blast cells, but a weak staining can be seen in a subset of B-ALL.[53] CD68 also belongs to the complementary panel useful for the characterization of plasmacytoid DCs,[19,45] which also includes the interleukin-3 receptor CD123 and DC differentiation antigens of the BDCA family.[19] CD123 is also expressed on a subset of B-ALL, where it can be used for the detection of MRD.[54] A subset of AML also expresses CD123, which makes this marker a possible hallmark of leukemia stem cells, and therefore a potential therapeutic target in $CD123^+$ cases.[55] In the rare cases associated with hyperbasophilia, the discriminant immunophenotype $CD123^+/DR^-$ will allow to identify this compartment.[56]

Finally, the marker 7.1 has been reported to be a hallmark of MLL rearrangements.[57]

These panels are intended for diagnostic purposes of AL. They may need to be completed with other markers that will prove later useful for MRD monitoring. This is especially true for crucial gating markers in AML such as CD11b and CD16 allowing to define and exclude mature normal cells.[24] In this context, CD58 for precursor B-ALL and CD99 for T-ALL could also be considered as useful for MRD follow-up.[58,59]

Since about two decades, the growing field of targeted therapies has revolutionized the treatment of hematological and other malignancies. Among other new drugs, a large place has been taken by monoclonal antibodies directed towards differentiation antigens, or toxin-conjugated ligands of surface molecules. It is therefore judicious to test for the expression these new therapeutic targets on blast cells from patients liable to enter clinical trials using or evaluating such drugs.[60] The list of useful markers to test at diagnosis can therefore be extended, according to current schedules applied in a given center, to include the well-known targets of rituximab (CD20),[61] or alemtuzumab (CD52),[62] but also CD45,[63] CD33,[64] CD116,[65] CD123,[55] CD44,[66] CD22[67] or CD87[68] as a non-exhaustive list.

## LPDs

Immunophenotyping for the diagnosis of LPD should be carried out to recognize features compatible with specific clinical questions, pointing the need to obtain relevant information as orientation toward the panel to consider. It has proven useful for screening and further classification into specific WHO entities (Supplementary Figure 2).

If the clinical question arises from absolute lymphocytosis or lymph node or skin involvement, a diagnosis of chronic lymphoid leukemia (CLL),[69] prolymphocytic leukemia, hairy cell leukemia (HCL)[70] or B-cell non-Hodgkin's lymphoma[71] should be evoked, although HCL may also be associated with leukopenia. Among B-cell lymphomas, the entities of Burkitt's, diffuse large B-cell lymphoma, follicular, mantle cell and marginal zone lymphoma should be the first to consider.

The possibility of facing a T-cell proliferative disorder, such as Sezary syndrome,[72] large granular lymphocyte lymphoma or another NK proliferative disorder, although much rarer in the Western world, should also be considered.[73]

## Mandatory panel

The mandatory panel proposed by ELN WP10 for LPD therefore comprises 23 markers. The strategies for choosing these markers have been delineated depending on the initial results of a gating or screening panel.

Immunophenotyping can be performed on peripheral blood, bone marrow, lymph node biopsy cell suspensions or occasionally ascites or cerebrospinal fluid. Fine-needle aspirations may be used for primary screening and to avoid unnecessary biopsies of reactive lymph nodes.[71,74]

It was agreed that three gating markers could be used to guide the subsequent steps of diagnosis. These would be CD19 as a hallmark of LPDs of the B lineage, CD3 for T lineage and CD56

for NK cells, although it should be noted that some of these disorders may be negative for these three markers. This combination would also provide rough but pertinent information as to the size of the remaining normal compartment of lymphocytes. Used on whole blood or whole bone marrow samples, eventually together with CD45, they will also allow to determine the proportion of the abnormal subset which, for example, in some cases of CLL, can be predominant.

After such screening, a B-lineage-oriented panel gated on CD19 was proposed (Supplementary Table 3). κ and λ expression will provide both an idea of the density of surface immunoglobulins (low or absent in CLL and in some cases of follicular lymphoma, overexpressed in other B-cell disorders) and a good approach to clonality. Search for these two markers and surface immunoglobulins should, however, be performed in Ficoll-separated or washed samples to avoid competition with soluble immunoglobulins. Other antigens will confirm B-lineage differentiation and allow further classification of the disorder. This is summarized in Supplementary Table 4, indicating the pertinence of CD5 co-expression in CLL and myeloid cell factor, of CD23 in CLL, of CD10 in FL and of CD103, CD25 and CD11c in HCL. A decreased expression or absence of CD22, CD81 and CD79b is also a hallmark of CLL cells, further segregating them from a residual component of normal B cells. Of note, prolymphocytic leukemias are typically composed of naïve μδ cells, with high CD79 and CD20 expression in the absence of CD23 and CD5.[75] The use of CD20 as a B-cell marker may, however, be improper for patients treated with monoclonal antibodies directed to that antigen.

The combination CD19/CD10/C38 can be useful to determine the size of the B-cell precursor population (hematogones) in bone marrow samples,[76] and it will also provide an approach to the possible presence of $CD19^{lo}/CD38^{+}$ plasma cells.[76,77]

It must be emphasized that Supplementary Table 3 reports the features most classically seen on B-cell LPDs. However, frontiers are leaky in this classification and atypical immunophenotypes are not rare. A close collaboration with pathologists and clinicians is mandatory to reach the most accurate diagnosis.

Gated on CD3 or another T-lineage marker, the T-lineage panel should comprise the following antigens: CD2, CD3, CD4, CD5, CD8 and CD7, mostly to investigate whether there is a decreased expression or absence (immunophenotypic 'holes') of one or more of these markers on a potentially clonal proliferation of T cells.[74]

The use of CD56 as a screening for potential $CD3^{-}/CD56^{+}$ proliferative disorders should be interpreted with caution, first because of the rarity of these disorders, and second because CD56 has a broader expression than just the NK lineage. A concomitant staining with CD45 could be useful in such cases to confirm the leukocytic nature of the CD56 compartment. It should be noted that a $CD56^{+}/CD45^{-}$ immunophenotype can be found on malignant plasma cells in myeloma and in bone marrow involvement of small round cell tumors of childhood such as neuroblastoma and rhabdomyosarcoma.[78]

### Additional useful markers

Depending either on clinical information or results of the primary screening as shown above, further markers can be considered. Investigating for FMC7 would allow full application of the Matutes' score[3] and provide further information as to the possible occurrence of HCL, splenic lymphoma with villous lymphocytes, marginal zone lymphoma or myeloid cell factor. In fact, the use of FMC7 in the mandatory panel could well be disputed. Differential labeling can be observed with CD43, upregulated in some B-cell disorders such as CLL.[79] CD200 and cyclin D1 may additionally help to discriminate B-CLL from mantle cell lymphoma in cases of B-NHL, although use of the latter marker in flow is still disputed.[80,81] CD123 is usually expressed on the HCL cells, which conversely may lack CD24.[69,82]

CD138 and CD38 expression are appropriate to detect cells engaged in plasmacytoid differentiation,[75] and this is the recommended combination, together with CD45, to gate plasma cells. A more detailed isotypic identification of surface or intracytoplasmic immunoglobulins, together with information on the presence of monoclonal protein in serum, may refine the diagnosis, for example, in cases of Waldenström macroglobulinemia.

CD81, a tetraspanin acting in conjunction with CD19 on normal B cells, is characteristically downregulated on CLL cells and, together with the absence of CD22, provides an excellent means of MRD follow-up in this disorder.[83]

CD10 is present in FL, in many cases of diffuse large B-cell lymphomas and in Burkitt's lymphoma as well as in B-cell precursor ALL; therefore, the final diagnosis should always be made in conjunction with morphology.[74]

Cytoplasmic Bcl2, CD38 and Zap70 can provide additional prognostic markers.[84]

Further investigation of T-lineage disorders can include a better exploration of the type of T-cell receptor expressed (αβ or γδ), especially as a good approach of clonality can be performed with a panel of Vβ specificities allowing to cover about 80% of the Vβ repertoire.[85] Evidence of the absence of CD7 expression on the $CD4^{+}$ cells of Sezary syndromes is extremely pertinent, and may be associated with the evidence of the absence of CD26, although this latter feature seems more controversial.[86,87] CD10 expression has also been reported on angioimmunoblastic T-cell lymphomas.[88]

Search for CD30 is of great value, associated with morphology and clinical features, in the diagnosis of Hodgkin's and anaplastic T-cell lymphomas.[89,90] However, the tumor cells in these entities may be difficult to detect by flow cytometry.

In addition to the precautions mentioned above for CD56 putative NK cell disorders, exploration of this lineage may benefit from the investigation of surface CD57, CD16, CD158 or CD94, and intracytoplasmic search for perforin or granzyme B.[91,92] The availability of a large range of reagents directed to NK receptors may also be useful to show the clonality of such very rare disorders.[93]

Finally, as for AL, therapeutic monoclonal antibodies are increasingly used in LPD, again not only directed to B cells but also in trials aimed at T-cell proliferations.[94]

This document provides the consensus collective view of participants of the ELN WP10 group as to the numbers and characteristics of markers necessary for a proper diagnosis of hematological malignancies in flow cytometry. The relevance of each of the suggested markers has been outlined and referenced, and is summarized in Supplementary Table 5. The mandatory panels lead to a correct diagnosis that would allow to properly direct therapy. Among the more than 360 clusters of differentiation depicting leukocyte antigens and the plethora of non-clustered molecules for which reagents are available, the markers selected by the WP10 group were chosen on the basis of experience and relevant literature. The list proposed in this document derives from the first that was posted in 2005, but has been updated. As other documents of this type,[27,28] it will evolve with time, but at the moment it provides an accurate state-of-the-art evaluation. The tremendous development of flow cytometry instruments and software in the past decades has

strengthened the pivotal role of this methodology in providing rapid and accurate information on immunophenotype, even on small samples or rare events by use of multidimensional approaches. Besides diagnosis, the follow-up of patients with hematological malignancies should benefit from this technology. Yet, such implementations will remain dependent on an accurate and comprehensive exploration at diagnosis, involving the pertinent choice of a large panel of antibodies justified by the heavy burden on the health system of these severe diseases. Screening strategies, as proposed with the orientation tube for LPD can be valuable. It may also not be necessary to type the cells in different samples. For instance, leukemic involvement of peripheral blood is usually providing pertinent information that need not be repeated on a bone marrow sample. The best compromise should be found between the benefit expected for the patient and the costs involved, yet a panel of around 30 antibodies can really be necessary in many instances. It is of health-economic importance to balance the relatively small cost of an appropriate comprehensive diagnostic immunophenotype against the tremendous burden on health-care systems brought by the treatment of these serious diseases.

The combinations to be used in each setting will depend on the technology available and are also in constant evolution. It is foreseeable that some degree of harmonization may be reached even considering the increasing complexity of multiparameter flow cytometry, but it is advisable that some freedom and flexibility remain. It is indeed important to continue investigating for ever more accurate and pertinent tests, providing clinicians and patients with prognostic information allowing to tailor induction and maintenance therapy as best as possible.

## Conflict of interest

The authors declare no conflict of interest.

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## References

1 http://www.leukemia-net.org/content/diagnostics/diagnostics (last accessed 20 November 2010).
2 Béné MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A *et al.* Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). *Leukemia* 1995; **9**: 1783–1786.
3 Moreau EJ, Matutes E, A'Hern RP, Morilla AM, Morilla RM, Owusu-Ankomah KA *et al.* Improvement of the chronic lymphocytic leukemia scoring system with the monoclonal antibody SN8 (CD79b). *Am J Clin Pathol* 1997; **108**: 378–382.
4 Dunphy CH. Contribution of flow cytometric immunophenotyping to the evaluation of tissues with suspected lymphoma? *Cytometry* 2000; **42**: 296–306.
5 Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J *et al.* The World Health Organization classification of neoplasms of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting—Airlie House, Virginia, November, 1997. *Hematol J* 2000; **1**: 53–66.
6 Béné MC, Kaeda JS. How and why minimal residual disease studies are necessary in leukemia: a review from WP10 and WP12 of the European Leukaemia Net. *Haematologica* 2009; **94**: 1135–1150.
7 Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H *et al.* eds *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. IARC: Lyon, France, 2008.
8 Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A *et al.* The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* 2009; **114**: 937–951.
9 Casasnovas RO, Slimane FK, Garand R, Faure GC, Campos L, Deneys V *et al.* Immunological classification of acute myeloblastic leukemias: relevance to patient outcome. *Leukemia* 2003; **17**: 515–527.
10 Lucio P, Gaipa G, van Lochem EG, van Wering ER, Porwit-MacDonald A, Faria T *et al.* BIOMED-I concerted action report: flow cytometric immunophenotyping of precursor B-ALL with standardized triple-stainings. BIOMED-1 Concerted Action Investigation of Minimal Residual Disease in Acute Leukemia: International Standardization and Clinical Evaluation. *Leukemia* 2001; **15**: 1185–1192.
11 Porwit-MacDonald A, Bjorklund E, Lucio P, van Lochem EG, Mazur J, Parreira A *et al.* BIOMED-1 concerted action report: flow cytometric characterization of CD7+ cell subsets in normal bone marrow as a basis for the diagnosis and follow-up of Tcell acute lymphoblastic leukemia (T-ALL). *Leukemia* 2000; **14**: 816–825.
12 Béné MC, Bernier M, Casasnovas RO, Castoldi G, Doekharan D, van der Holt B *et al.* Myeloid leukaemia M0: haematological, immunophenotypic and cytogenetic characteristics and their prognostic significance: an analysis in 241 patients. *Br J Haematol* 2001; **113**: 737–745.
13 Tallman MS. Relevance of pathologic classifications and diagnosis of acute myeloid leukemia to clinical trials and clinical practice. *Cancer Treat Res* 2004; **121**: 45–67.
14 Paietta E. Expression of cell-surface antigens in acute promyelocytic leukaemia. *Best Pract Res Clin Haematol* 2003; **16**: 369–385.
15 Domingo-Claros A, Larriba I, Rozman M, Irriguible D, Vallespí T, Aventin A *et al.* Acute erythroid neoplastic proliferations. A biological study based on 62 patients. *Haematologica* 2002; **87**: 148–153.
16 Duchayne E, Fenneteau O, Pages MP, Sainty D, Arnoulet C, Dastugue N *et al.* Groupe Francais d'Hematologie Cellulaire; Groupe Francais de Cytogenetique Hematologique. Acute megakaryoblastic leukaemia: a national clinical and biological study of 53 adult and childhood cases by the Groupe Francais d'Hematologie Cellulaire (GFHC). *Leuk Lymphoma* 2003; **44**: 49–58.
17 Jacob MC, Chaperot L, Mossuz P, Feuillard J, Valensi F, Leroux D *et al.* CD4+ CD56+ lineage negative malignancies: a new entity developed from malignant early plasmacytoid dendritic cells. *Haematologica* 2003; **88**: 941–955.
18 Béné MC, Feuillard J, Jacob MC. Groupe d'Etude Immunologique des Leucemies. Plasmacytoid dendritic cells: from the plasmacytoid T-cell to type 2 dendritic cells CD4+CD56+ malignancies. *Semin Hematol* 2003; **40**: 257–266.
19 Garnache-Ottou F, Feuillard J, Ferrand C, Biichle S, Trimoreau F, Seilles E *et al.* GOELAMS and GEIL study. Extended diagnostic criteria for plasmacytoid dendritic cell leukaemia. *Br J Haematol* 2009; **145**: 624–636.
20 Duchayne E, Demur C, Rubie H, Robert A, Dastugue N. Diagnosis of acute basophilic leukemia. *Leuk Lymphoma* 1999; **32**: 269–278.
21 Valent P, Samorapoompichit P, Sperr WR, Horny HP, Lechner K. Myelomastocytic leukemia: myeloid neoplasm characterized by partial differentiation of mast cell-lineage cells. *Hematol J* 2002; **3**: 90–94.
22 Lacombe F, Durrieu F, Briais A, Dumain P, Belloc F, Bascans E *et al.* Flow cytometry CD45 gating for immunophenotyping of acute myeloid leukemia. *Leukemia* 1997; **11**: 1878–1886.
23 Borowitz MJ, Guenther KL, Shults KE, Stelzer GT. Immunophenotyping of acute leukemia by flow cytometric analysis. Use of CD45 and right-angle light scatter to gate on leukemic blasts in three-color analysis. *Am J Clin Pathol* 1993; **100**: 534–540.
24 Lacombe F, Arnoulet C, Maynadié M, Lippert E, Luquet I, Pigneux A *et al.* Early clearance of peripheral blasts measured by flow cytometry during the first week of AML induction therapy as a new independent prognostic factor: a GOELAMS study. *Leukemia* 2009; **23**: 350–357.
25 Arnoulet C, Béné MC, Durrieu F, Feuillard J, Fossat C, Husson B *et al.* Four- and five-color flow cytometry analysis of leukocyte

differentiation pathways in normal bone marrow: a reference document based on a systematic approach by the GTLLF and GEIL. *Cytometry B* 2010; **78**: 4–10.
26 http://www.leukemia-net.org/content/diagnostics/diagnostics/flow_cytometry_atlas/last (last accessed 20 November 2010).
27 Basso G, Buldini B, De Zen L, Orfao A. New methodologic approaches for immunophenotyping acute leukemias. *Haematologica* 2001; **86**: 675–692.
28 Stetler-Stevenson M, Davis B, Wood B, Braylan R. 2006 Bethesda international consensus conference on flow cytometric immunophenotyping of hematolymphoid neoplasia. *Cytometry B* 2007; **72** (Suppl 1): S3.
29 Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. *Blood* 2008; **111**: 3941–3967.
30 Karube K, Aoki R, Nomura Y, Yamamoto K, Shimizu K, Yoshida S *et al.* Usefulness of flow cytometry for differential diagnosis of precursor and peripheral T-cell and NK-cell lymphomas: analysis of 490 cases. *Pathol Int* 2008; **58**: 89–97.
31 Al-Mawali A, Gillis D, Hissaria P, Lewis I. Incidence, sensitivity, and specificity of leukemia-associated phenotypes in acute myeloid leukemia using specific five-color multiparameter flow cytometry. *Am J Clin Pathol* 2008; **129**: 934–945.
32 Sato N, Kishi K, Toba K, Watanabe K, Itoh H, Narita M *et al.* Simultaneous expression of CD13, CD22 and CD25 is related to the expression of Fc epsilon R1 in non-lymphoid leukemia. *Leuk Res* 2004; **28**: 691–698.
33 Béné MC, Faure GC. CD10 in acute leukemias. GEIL. *Haematologica* 1997; **82**: 205–210.
34 Herzog S, Reth M, Jumaa H. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. *Nat Rev Immunol* 2009; **9**: 195–205.
35 Béné MC, Bernier M, Casasnovas RO, Castoldi G, Knapp W, Lanza F *et al.* The reliability and specificity of c-kit for the diagnosis of acute myeloid leukemias and undifferentiated leukemias. The European Group for the Immunological Classification of Leukemias (EGIL). *Blood* 1998; **92**: 596–599.
36 Maynadié M, Campos L, Moskovtchenko P, Sabido O, Aho S, Lenormand B *et al.* GEIL. Heterogenous expression of CD15 in acute lymphoblastic leukemia: a study of ten anti-CD15 monoclonal antibodies in 158 patients. *Leuk Lymphoma* 1997; **25**: 135–143.
37 Betz SA, Foucar K, Head DR, Chen IM, Willman CL. False-positive flow cytometric platelet glycoprotein IIb/IIIa expression in myeloid leukemias secondary to platelet adherence to blasts. *Blood* 1992; **79**: 2399–2403.
38 Babusíková O, Stevulová L. Analysis of surface and cytoplasmic immunoglobulin light/heavy chains by flow cytometry using a lysed-whole-blood technique: Implications for the differential diagnosis of B-cell malignancies. *Neoplasma* 2004; **51**: 422–430.
39 Gandemer V, Aubry M, Roussel M, Rio AG, de Tayrac M, Vallee A *et al.* CD9 expression can be used to predict childhood TEL/AML1-positive acute lymphoblastic leukemia: Proposal for an accelerated diagnostic flowchart. *Leuk Res* 2010; **34**: 430–437.
40 Tiacci E, Pileri S, Orleth A, Pacini R, Tabarrini A, Frenguelli F *et al.* PAX5 expression in acute leukemias: higher B-lineage specificity than CD79a and selective association with t(8;21)-acute myelogenous leukemia. *Cancer Res* 2004; **64**: 7399–7404.
41 Schott G, Sperling C, Schrappe M, Ratei R, Martin M, Meyer U *et al.* Immunophenotypic and clinical features of T-cell receptor (TCR) γδ+ T-lineage acute lymphoblastic leukemia (T-ALL). *Br J Haematol* 1998; **101**: 753–755.
42 Strobl H, Knapp W. Myeloid cell-associated lysosomal proteins as flow cytometry markers for leukocyte lineage classification. *J Biol Regul Homeost Agents* 2004; **18**: 335–339.
43 Knapp W, Strobl H, Majdic O. Flow cytometric analysis of cell-surface and intracellular antigens in leukemia diagnosis. *Cytometry* 1994; **18**: 187–198.
44 Scholz W, Platzer B, Schumich A, Hocher B, Fritsch G, Knapp W *et al.* Initial human myeloid/dendritic cell progenitors identified by absence of myeloperoxidase protein expression. *Exp Hematol* 2004; **32**: 270–276.
45 Scheinecker C, Strobl H, Fritsch G, Csmarits B, Krieger O, Majdic O *et al.* Granulomonocyte-associated lysosomal protein expression during *in vitro* expansion and differentiation of CD34+ hematopoietic progenitor cells. *Blood* 1995; **86**: 4115–4123.
46 Strobl H, Scheinecker C, Riedl E, Csmarits B, Bello-Fernandez C, Pickl WF *et al.* Identification of CD68+lin− peripheral blood cells with dendritic precursor characteristics. *J Immunol* 1998; **161**: 740–748.
47 Wuchter C, Ratei R, Spahn G, Schoch C, Harbott J, Schnittger S *et al.* Impact of CD133 (AC133) and CD90 expression analysis for acute leukemia immunophenotyping. *Haematologica* 2001; **86**: 154–161.
48 Noto R, Lo Pardo C, Schiavone EM, Ferrara F, Manzo C, Vacca C *et al.* All-trans retinoic acid (ATRA) and the regulation of adhesion molecules in acute myeloid leukemia. *Leuk Lymphoma* 1996; **21**: 201–209.
49 Lee JJ, Cho D, Chung IJ, Cho SH, Park KS, Park MR *et al.* CD34 expression is associated with poor clinical outcome in patients with acute promyelocytic leukemia. *Am J Hematol* 2003; **73**: 149–153.
50 Braylan RC, Orfao A, Borowitz MJ, Davis BH. Optimal number of reagents required to evaluate hematolymphoid neoplasias: results of an international consensus meeting. *Cytometry* 2001; **46**: 23–27.
51 Nakahata T, Okumura N. Cell surface antigen expression in human erythroid progenitors: erythroid and megakaryocytic markers. *Leuk Lymphoma* 1994; **13**: 401–409.
52 Garand R, Duchayne E, Blanchard D, Robillard N, Kuhlein E, Fenneteau O *et al.* Minimally differentiated erythroleukaemia (AML M6 'variant'): a rare subset of AML distinct from AML M6. Groupe Francais d'Hematologie Cellulaire. *Br J Haematol* 1995; **90**: 868–875.
53 Strobl H, Scheinecker C, Csmarits B, Majdic O, Knapp W. Flow cytometric analysis of intracellular CD68 molecule expression in normal and malignant haemopoiesis. *Br J Haematol* 1995; **90**: 774–782.
54 Djokic M, Björklund E, Blennow E, Mazur J, Söderhäll S, Porwit A. Overexpression of CD123 correlates with the hyperdiploid genotype in acute lymphoblastic leukemia. *Haematologica* 2009; **94**: 1016–1019.
55 Jin L, Lee EM, Ramshaw HS, Busfield SJ, Peoppl AG, Wilkinson L *et al.* Monoclonal antibody-mediated targeting of CD123, IL-3 receptor alpha chain, eliminates human acute myeloid leukemic stem cells. *Cell Stem Cell* 2009; **5**: 31–42.
56 Han X, Jorgensen JL, Brahmandam A, Schlette E, Huh YO, Shi Y *et al.* Immunophenotypic study of basophils by multiparameter flow cytometry. *Arch Pathol Lab Med* 2008; **132**: 813–819.
57 Wuchter C, Harbott J, Schoch C, Schnittger S, Borkhardt A, Karawajew L *et al.* Detection of acute leukemia cells with mixed lineage leukemia (MLL) gene rearrangements by flow cytometry using monoclonal antibody 7.1. *Leukemia* 2000; **14**: 1232–1238.
58 Veltroni M, De Zen L, Sanzari MC, Maglia O, Dworzak MN, Ratei R *et al.* I-BFM-ALL-FCM-MRD-Study Group. Expression of CD58 in normal, regenerating and leukemic bone marrow B cells: implications for the detection of minimal residual disease in acute lymphocytic leukemia. *Haematologica* 2003; **88**: 1245–1252.
59 Dworzak MN, Fröschl G, Printz D, Zen LD, Gaipa G, Ratei R *et al.* CD99 expression in T-lineage ALL: implications for flow cytometric detection of minimal residual disease. *Leukemia* 2004; **18**: 703–708.
60 Morris JC, Waldmann TA. Antibody-based therapy of leukaemia. *Expert Rev Mol Med* 2009; **11**: e29.
61 Thomas DA, O'Brien S, Kantarjian HM. Monoclonal antibody therapy with rituximab for acute lymphoblastic leukemia. *Hematol Oncol Clin N Am* 2009; **23**: 949–971.
62 Angiolillo AL, Yu AL, Reaman G, Ingle AM, Secola R, Adamson PC. A phase II study of Campath-1H in children with relapsed or refractory acute lymphoblastic leukemia: a Children's Oncology Group report. *Pediatr Blood Cancer* 2009; **53**: 978–983.
63 Pagel JM, Gooley TA, Rajendran J, Fisher DR, Wilson WA, Sandmaier BM *et al.* Allogeneic hematopoietic cell transplantation after conditioning with $^{131}$I-anti-CD45 antibody plus fludarabine and low-dose total body irradiation for elderly patients with advanced acute myeloid leukemia or high-risk myelodysplastic syndrome. *Blood* 2009; **114**: 5444–5453.
64 Stasi R. Gemtuzumab ozogamicin: an anti-CD33 immunoconjugate for the treatment of acute myeloid leukaemia. *Expert Opin Biol Ther* 2008; **8**: 527–540.

65 Frankel AE, Powell BL, Hall PD, Case LD, Kreitman RJ. Phase I trial of a novel diphtheria toxin/granulocyte macrophage colony-stimulating factor fusion protein (DT388GMCSF) for refractory or relapsed acute myeloid leukemia. *Clin Cancer Res* 2002; **8**: 1004–1013.
66 Jin L, Hope KJ, Zhai Q, Smadja-Joffe F, Dick JE. Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nat Med* 2006; **12**: 1167–1174.
67 Stanciu-Herrera C, Morgan C, Herrera L. Anti-CD19 and anti-CD22 monoclonal antibodies increase the effectiveness of chemotherapy in pre-B acute lymphoblastic leukemia cell lines. *Leuk Res* 2008; **32**: 625–632.
68 Ramage JG, Vallera DA, Black JH, Aplan PD, Kees UR, Frankel AE. The diphtheria toxin/urokinase fusion protein (DTAT) is selectively toxic to CD87 expressing leukemic cells. *Leuk Res* 2003; **27**: 79–84.
69 Matutes E, Polliack A. Morphological and immunophenotypic features of chronic lymphocytic leukemia. *Rev Clin Exp Hematol* 2000; **4**: 22–47.
70 Del Giudice I, Matutes E, Morilla R, Morilla A, Owusu-Ankomah K, Rafiq F *et al*. The diagnostic value of CD123 in B-cell disorders with hairy or villous lymphocytes. *Haematologica* 2004; **89**: 303–308.
71 Laane E, Tani E, Bjorklund E, Elmberger G, Everaus H, Skoog L *et al*. Flow cytometric immunophenotyping including Bcl-2 detection on fine needle aspirates in the diagnosis of reactive lymphadenopathy and non-Hodgkin's lymphoma. *Cytometry B* 2005; **64**: 34–42.
72 Matutes E. Chronic T-cell lymphoproliferative disorders. *Rev Clin Exp Hematol* 2002; **6**: 401–420.
73 Lundell R, Hartung L, Hill S, Perkins SL, Bahler DW. T-cell large granular lymphocyte leukemias have multiple phenotypic abnormalities involving pan-T-cell antigens and receptors for MHC molecules. *Am J Clin Pathol* 2005; **124**: 937–946.
74 Jorgensen JL. State of the art symposium: flow cytometry in the diagnosis of lymphoproliferative disorders by fine-needle aspiration. *Cancer* 2005; **105**: 443–451.
75 Bettelheim P, Diem H, Nebe T. Flow cytometric typing of B-cell non Hodgkin lymphoma. *Lab Med* 2004; **28**: 410–423.
76 McKenna RW, Asplund SL, Kroft SH. Immunophenotypic analysis of hematogones (B-lymphocyte precursors) and neoplastic lymphoblasts by 4-color flow cytometry. *Leuk Lymphoma* 2004; **45**: 277–285.
77 Bataille R, Jégo G, Robillard N, Barillé-Nion S, Harousseau JL, Moreau P *et al*. The phenotype of normal, reactive and malignant plasma cells. Identification of 'many and multiple myelomas' and of new targets for myeloma therapy. *Haematologica* 2006; **91**: 1234–1240.
78 Bozzi F, Collini P, Aiello A, Barzanò E, Gambirasio F, Podda M *et al*. Flow cytometric phenotype of rhabdomyosarcoma bone marrow metastatic cells and its implication in differential diagnosis with neuroblastoma. *Anticancer Res* 2008; **28**: 1565–1569.
79 Jung G, Eisenmann JC, Thiébault S, Hénon P. Cell surface CD43 determination improves diagnostic precision in late B-cell diseases. *Br J Haematol* 2003; **120**: 496–499.
80 Palumbo GA, Parrinello N, Fargione G, Cardillo K, Chiarenza A, Berretta S *et al*. CD200 expression may help in differential diagnosis between mantle cell lymphoma and B-cell chronic lymphocytic leukemia. *Leuk Res* 2009; **33**: 1212–1216.
81 Jain P, Giustolisi GM, Atkinson S, Elnenaei MO, Morilla R, Owusu-Ankomah K *et al*. Detection of cyclin D1 in B cell lymphoproliferative disorders by flow cytometry. *J Clin Pathol* 2002; **55**: 940–945.
82 Matutes E, Morilla R, Owusu-Ankomah K, Houliham A, Meeus P, Catovsky D. The immunophenotype of hairy cell leukemia (HCL). Proposal for a scoring system to distinguish HCL from B-cell disorders with hairy or villous lymphocytes. *Leuk Lymphoma* 1994; **14**(Suppl 1): 57–61.
83 Rawstron AC, Villamor N, Ritgen M, Böttcher S, Ghia P, Zehnder JL *et al*. International standardized approach for flow cytometric residual disease monitoring in chronic lymphocytic leukaemia. *Leukemia* 2007; **21**: 956–964.
84 Kern W, Dicker F, Schnittger S, Haferlach C, Haferlach T. Correlation of flow cytometrically determined expression of ZAP-70 using the SBZAP antibody with IgVH mutation status and cytogenetics in 1,229 patients with chronic lymphocytic leukemia. *Cytometry B* 2009; **76**: 385–393.
85 Salameire D, Le Bris Y, Fabre B, Fauconnier J, Solly F, Pernollet M *et al*. Efficient characterization of the TCR repertoire in lymph nodes by flow cytometry. *Cytometry A* 2009; **75**: 743–751.
86 Kelemen K, Guitart J, Kuzel TM, Goolsby CL, Peterson LC. The usefulness of CD26 in flow cytometric analysis of peripheral blood in Sézary syndrome. *Am J Clin Pathol* 2008; **129**: 146–156;.
87 Sokolowska-Wojdylo M, Wenzel J, Gaffal E, Steitz J, Roszkiewicz J, Bieber T *et al*. Absence of CD26 expression on skin-homing CLA+ CD4+ T lymphocytes in peripheral blood is a highly sensitive marker for early diagnosis and therapeutic monitoring of patients with Sézary syndrome. *Clin Exp Dermatol* 2005; **30**: 702–706.
88 Stacchini A, Demurtas A, Aliberti S, Francia di Celle P, Godio L, Palestro G *et al*. The usefulness of flow cytometric CD10 detection in the differential diagnosis of peripheral T-cell lymphomas. *Am J Clin Pathol* 2007; **128**: 854–864.
89 Lee PS, Lin CN, Chuang SS. Immunophenotyping of angioimmunoblastic T-cell lymphomas by multiparameter flowcytometry. *Pathol Res Pract* 2003; **199**: 539–545.
90 Fromm JR, Thomas A, Wood BL. Flow cytometry can diagnose classical Hodgkin lymphoma in lymph nodes with high sensitivity and specificity. *Am J Clin Pathol* 2009; **131**: 322–332.
91 Akiyama T, Okino T, Konishi H, Wani Y, Notohara K, Tsukayama C *et al*. CD8+, CD56+ (natural killer-like) T-cell lymphoma involving the small intestine with no evidence of enteropathy: clinicopathology and molecular study of five Japanese patients. *Pathol Int* 2008; **58**: 626–634.
92 Fischer L, Hummel M, Burmeister T, Schwartz S, Thiel E. Skewed expression of natural-killer (NK)-associated antigens on lymphoproliferations of large granular lymphocytes (LGL). *Hematol Oncol* 2006; **24**: 78–85.
93 Chiesa S, Tomasello E, Vivier E, Vély F. Coordination of activating and inhibitory signals in natural killer cells. *Mol Immunol* 2005; **42**: 477–484.
94 Morris JC, Waldmann TA, Janik JE. Receptor-directed therapy of T-cell leukemias and lymphomas. *J Immunotoxicol* 2008; **5**: 235–248.

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